Title: Mechanisms of reactivation of latent tuberculosis infection due to SIV co-infection

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Abstract.

HIV is a major driver of Tuberculosis (TB) reactivation. Depletion of CD4+ T cells is assumed to be the basis behind TB reactivation in individuals with latent tuberculosis Infection (LTBI) co-infected with human immunodeficiency virus (HIV). Non-human primates (NHPs) coinfected with a mutant simian immunodeficiency virus (SIVΔGY), that does not cause depletion of tissue CD4+ T cells during infection, failed to reactivate TB. To investigate the contribution of CD4+ T cell depletion relative to other mechanisms of SIV-induced reactivation of LTBI, we used CD4R1 antibody to deplete CD4+ T cells in animals with LTBI without lentiviral infection. The mere depletion of CD4+ T cells during LTBI was insufficient in generating reactivation of LTBI. Instead, direct cytopathic effects of SIV resulting in chronic immune activation, along with the altered effector T cell phenotypes and dysregulated T cell homeostasis, were likely mediators of reactivation of LTBI. These results revealed important implications for TB control in HIV co-infected individuals.
Introduction.

Tuberculosis (TB) and HIV co-infection are a global syndemic (1). ~90% of HIV-negative *Mtb*-infected individuals lack clinical signs of TB and are considered to have Latent TB infection (LTBI) (1, 2). Although, the immune systems of exposed individuals successfully contain *Mtb* infection in most individuals, bacteria can persist within lung granulomas for long periods before reactivating to TB disease (3, 4). We seek to understand the mechanisms by which HIV co-infection reactivates TB using the *Mtb*/Simian Immunodeficiency Virus (SIV) co-infection model in rhesus macaques.

CD4+ T cell depletion is a hallmark of HIV and SIV infection and is widely regarded as the primary cause of immunodeficiency. Previously, we identified *Mtb*/SIV co-infected primates that did not progress to TB despite productive co-infection, raising the possibility of CD4+ T cell-independent mechanisms of LTBI control (5). To investigate the contribution of CD4+ T cell depletion relative to other mechanisms of SIV-induced reactivation of LTBI, we used antibodies to deplete CD4+ T cells and compared the outcomes to coinfection with pathogenic SIV and the mutant virus, SIVΔGY. Co-infection of *Mtb* with pathogenic SIV, but neither the mutant nor antibody-mediated CD4+ T cell depletion, resulted in reactivation.

Results and Discussion.
To assess the role of lung CD4\(^+\) T cells in protecting against reactivation of LTBI, 40 Indian rhesus macaques were exposed to low-dose aerosol *Mycobacterium tuberculosis* (*Mtb*) infection to establish TB latency (Fig 1A, Supplementary Table 1). At week 9 post-TB infection, a subset of 16 macaques experiencing asymptomatic LTBI were co-infected with pathogenic SIVmac239. These animals exhibited two distinct outcomes: 9 animals developed signs of reactivated TB (reactivators) while 7 retained *Mtb* latency despite productive SIV infection and peripheral blood viremia (nonreactivators) (5). To investigate the role of CD4\(^+\) T cells in our low dose aerosol model, we co-infected 6 macaques with a novel variant of pathogenic SIVmac29 molecular clone, SIVmac239ΔGY (SIVΔGY) (6), in which a deletion of two amino acids from a trafficking motif in the envelope gp41 cytoplasmic domain leads to viral replication, but does not deplete CD4\(^+\) T cells in the periphery or in the lamina propria (7) (Supplementary Table 1). In addition, we used antibody-mediated depletion of CD4\(^+\) T cells in 8 macaques with LTBI using CD4R1, which was administered every two weeks after week 9 post-*Mtb* infection (Fig 1A, Supplementary Table 1).

Importantly, LTBI/SIVΔGY co-infected and CD4R1-administered macaques retained control of TB similar to nonreactivators. Specifically, only 1/8 CD4R1-administered NHPs displayed symptomatology consistent with reactivated TB that necessitated a humane necropsy (Fig 1). *Mtb*/SIVΔGY co-infected and CD4R1-administered macaques showed normal serum CRP levels over time (Fig S1A) and at
endpoint (Fig 1B), comparable to LTBI and nonreactivators and statistically different from reactivators. These animals maintained relatively normal body temperatures (Fig 1C) and weights (Fig 1D). SIVΔGY co-infected and CD4R1-administered NHPs had lower numbers of viable Mtb in their bronchoalveolar lavage (BAL) fluid throughout infection (Fig S1B), and significantly lower viable Mtb in their BAL at endpoint (Fig 1F). Similarly, both experimental groups harbored low lung (Fig 1G), bronchial lymph node (Fig 1H), spleen (Fig S1C), liver (Fig S1D), and kidney (Fig S1E) bacterial burdens, comparable to the LTBI and nonreactivators. Both experimental groups possessed significantly lower viable Mtb in all tissues at necropsy compared to reactivators. Finally, virtually no tuberculous lung pathology was observed in Mtb/SIVΔGY co-infected NHPs, demonstrating that co-infection with this virus failed to reactivate LTBI (Fig 1I-J). 1/8 CD4R1-administered NHPs with LTBI did reactivate, displaying an elevated CRP at necropsy (Fig 1B) and CXR score (Fig 1I). Measurement of peripheral viremia in co-infected animals suggested that SIVΔGY replicated to comparable levels in the acute phase and established similar set points (Fig 1K). Although significantly lower peripheral viremia was observed at peak in our SIVΔGY-coinfected NHPs compared to SIVmac239-coinfected reactivators and non-reactivators, this is not unexpected as rhesus macaques infected SIVΔGY often have variable viremia (8, 9).

NHPs with LTBI/SIVΔGY co-infection did not exhibit a significant decline in CD4+ T cell levels in peripheral blood (Fig 2A, Fig S2A, FigS2C) or BAL (Fig 2B, Fig S2B, Fig
S2D). This was in stark contrast to animals infected with pathogenic SIV (Fig 2A-B), consistent with previous results (5, 10). Although a significant reduction in CD4+ T cells was observed in the lungs (Fig 2C) of LTBI/SIVΔGY co-infected NHPs, an insignificant reduction was observed in the total CD4+ T cell compartment (Fig S2E). Previously, SIVΔGY had been shown to replicate in the plasma and lymphoid tissues, but to spare gut mucosal tissues (7, 8). To our knowledge, this was the first time evaluation of lung CD4+ T cell populations in SIVΔGY-infected NHPs, so this was a novel finding. There may be sufficient lymphoid tissue in the lungs that SIVΔGY was able to replicate nearby, perhaps in inducible bronchus-associated lymphoid tissue (iBALT) (5), which led to CD4+ T cell depletion during SIVΔGY-coinfection. Significant depletion of CD4+ T cells was observed in the peripheral blood (Fig 2D), BAL (Fig 2E), and lungs (Fig 2F) of CD4R1-administered NHPs, relative to LTBI and SIVΔGY co-infected NHPs. The levels of CD4-depletion were comparable to SIVmac239-infected animals and the differences in CD4+ T cell populations between NHPs administered CD4R1 and NHPs co-infected with SIVmac239 were not significant in the BAL or lungs (Fig 2E-F), regardless of their TB outcome. This result was confirmed by multilabel confocal immunofluorescence microscopy (Fig 2G-K). Thus, our results show that co-infection of macaques with LTBI with a nonpathogenic virus that fails to deplete CD4+ T cells, SIVΔGY, and depletion of CD4+ T cells using antibodies does not result in similar rates of TB reactivation observed upon co-infection with pathogenic SIVmac239.
As early as week 2 post-SIV challenge or post-CD4R1 administration, NHPs displayed significant changes in the distribution of their residual CD4+ T cells. *Mtb* infection alone can impact the distribution of effector and memory cells, and memory CD4+ T cells are associated with protection (11-13). We compared three groups of NHPs, those with LTBI to NHPs with CD4+ T cell depletion due to SIVmac239 co-infection and CD4R1 administration. Although CD4+ T cells were massively depleted in both experimental groups of NHPs, it was vital to understand the contribution of the remaining CD4+ T cells to the host’s immune defenses. Co-infection with SIVmac239 resulted in a significant reduction of memory (CD95+CD28+) CD4+ T cells, which were replaced by naïve (CD95−CD28+), not effector (CD95−CD28+), CD4+ T cells (Fig 3A). Most remaining CD4+ T cells in the blood of SIV co-infected animals were naïve, consistent with findings that Mtb/HIV co-infected patients harbor less differentiated CD4+ memory T cells compared to TB-only patients (14). HIV infection can lead to an activated and exhausted memory CD4+ T cell phenotype (15), which likely contributed to the more significant loss of memory CD4+ T cells. Compared to animals with LTBI, CD4R1-administered NHPs exhibited significantly higher frequencies of effector and reduced naïve and memory CD4+ T cells in the peripheral blood (Fig 3A). In the peripheral blood, CD8+ T cells did not differ between groups (Fig 3B). In the BAL, changes were observed only in the CD4+ (Fig 3C) and not the CD8+ T cell compartment (Fig 3D). Memory CD4+ T cells were replaced by naïve cells upon SIV co-infection in the BAL (Fig 3C). Interestingly, the distribution of
BAL CD4+ T cells following CD4-depletion by SIVmac239 co-infection did not significantly differ from the distributions observed during LTBI, indicating that the virus affected all compartments equally within the BAL. BAL effector CD4+ T cell populations were significantly increased in CD4R1-administered NHPs compared to both LTBI and SIVmac239-coinfected NHPs, while memory CD4+ T cell populations were significantly reduced (Fig 3C). Throughout CD4R1 administration, CD4+ T cells in the BAL reflected a bias towards effector (Fig 3E) rather than memory (Fig 3F) populations. Greater proportions of effector T cells in the lungs of CD4R1-administered, versus SIV co-infected, NHPs strongly suggest that early pathogenic events associated with SIV co-infection suppress the natural homeostatically-driven CD4+ T cell proliferation and downstream effector T cell functions.

Gene-expression analyses in the BAL from SIV co-infected versus CD4R1-administered NHPs confirmed our assertion that CD4+ T cell maturation states differed in these two groups. Real-time RT-PCR analysis revealed that at week 11 and 15 post-Mtb, NHPs administered CD4R1 had significantly higher levels of transcripts involved in T cell activation, differentiation, and function, e.g., IL-2 (key signal for Th1 differentiation), IL-12B (important for sustaining memory/effector Th1 responses against Mtb), and IL-17A (expressed on Th17+ cells), compared to SIV reactivators (Fig 3G). These cytokines indicate that although CD4R1 depleted the CD4+ T cell compartment, it did not
inhibit T cell maturation and signaling. On the contrary, SIV co-infection inhibited T cell maturation and signaling.

Gene-enrichment analyses on lung transcriptome confirmed that SIV co-infected reactivators experienced chronic immune activation and a cytokine storm. CD4+ T cells homeostasis was enhanced in CD4R1-administered NHPs and unperturbed during SIV co-infection. Gene terms ‘Immunoglobulin/Major Histocompatibility Complex (MHC),’ ‘MHC Class I/Class II antigen recognition protein,’ etc. were significantly enriched only in CD4R1-administered NHPs (Fig 4A, Supplementary Table 2, InterPro). Several Categories encoding proteins interacting with RAS (Fig 4A), involved in cell signaling pathways that control cell growth and cell death, were only significantly enriched in CD4R1-administered NHPs. This underscores that normal immune homeostasis is observed in CD4R1-administered NHPs, despite severe lymphopenia. Categories like ‘Inflammatory Response,’ ‘Innate Immunity,’ and ‘Endoplasmic Reticulum’ were only enriched in SIV reactivators, suggesting chronic immune activation and an ensuing cytokine storm in that group (Fig 4B). Unsurprisingly, SIV reactivators were characterized by the enrichment of gene categories corresponding to chronic immune activation and inflammation including ‘TNF-signaling’ and ‘Fc-gamma receptor mediated phagocytosis’ (Fig 4C). Reactivator lungs significantly expressed high levels of genes involved in proinflammatory cytokine/chemokine signaling (CXCL12, IL6), myeloid cell/phagocyte/complement/innate immune signaling (CD68, CFH, C1S,
TXNDC11), matrix degradation critical for granuloma formation (IGFBP7), and antigen presentation (HLAB) (Supplementary Table 2). Genes belonging to the pathway ‘regulation of lymphocyte differentiation,’ including NDFIP1, FAS, and RNF41, were induced in lungs of reactivators. The genes only expressed in nonreactivators’ lung included those involved in the restoration of normal lung and cytoskeletal function (WWOX, TRIOBP, PEAK3), B cell function (SPANXA1) (16), repair of acute tissue injury (MYLK2) (17), inhibition of cytokine storm (TIP39, SLC8A2) (18), viral restriction (APOBEC3) (19), induction of apoptosis (IFT57), and induction of robust cellular immune responses (CLECB15/KLRG2, BTN2A3, TSPEAR, MRAP).

Real time RT-PCR and multiplex cytokine analysis strongly confirmed genome-wide transcriptome results. Cytokines associated with inflammation, including TNF-α, IL1-α, IL-1β and IL-6, were highly induced in the lungs of SIV co-infected reactivators compared to CD4R1-administered NHPs (Fig 4D). Multiplex analysis supported this trend, showing that in the BAL supernatant at necropsy, TNF-α, IL1-α, IL-1β and IL-6 proteins were more abundant in SIV co-infected reactivators (Fig 4E). Significantly increased IFN-γ was detected in the plasma (Fig S4A) and BAL (Fig S4B) of reactivators compared to other groups. Other markers of increased cell signaling and migration associated with inflammatory responses were significantly increased in the plasma (CXCL9; Fig S4H) or BAL (CXCL13; Fig S4C, eotaxin; Fig S4E, I-TAC; Fig S4G, and IL-6; Fig S4J) of reactivators.
Chronic immune activation in SIV co-infected animals was supported by immunophenotyping. T cells in the lungs of reactivators had a significantly more activated phenotype (HLA-DR+) compared to those from NHPs that received CD4R1 (Fig 4F). HLA-DR+ (Fig 4G) and HLA-DR− (Fig 4H) T cells in reactivators exhibited significantly higher CCR5 (recruitment) and CD69 (early activation) markers compared to CD4R1 administration. This suggests higher recruitment and entrapment of activated lymphocytes at the primary site of infection in reactivators. CCR5-associated recruitment to the immune synapse induces stronger T cell–APC attraction that results in reduced T cell responsiveness to chemokines (20). This response is typically followed by increased T cell proliferation and upregulated effector responses (20, 21), which was observed through increased CD69 expression by SIV reactivators. Furthermore, CCR7+ T cell populations were significantly reduced in reactivators compared to CD4R1-administered NHPs (Fig 4G-H), suggesting that migration to secondary lymph nodes was impaired in T cells from the lungs of reactivators (22). Significantly reduced PD-1 expression in reactivators compared to CD4R1-administered NHPs indicated that HLA-DR+ (Fig 4G), but not HLA-DR− (Fig 4H), T cells in reactivators were subject to regulation, strongly supporting a pattern of chronic immune activation in reactivators. As this pattern was also observed in the CD8+ T cell compartment (Fig S5A-C), SIV-mediated CD4+ T cell depletion is likely not the sole cause of immune imbalance leading to chronic activation as the T cell recruitment, activation and homeostatic regulation was maintained in NHPs.
with antibody-mediated CD4+ T cell depletion. As can be seen in the reactivators with reduced PD-1 expression, some of these inhibitory responses are not being maintained, thus allowing increased immune activation and the cytokine storm seen in our transcriptomcis data to occur.

Although it is known that the functions of mycobacterial-specific CD4+ T cells are impaired during HIV-infection, the immunological mechanisms underlying how CD4+ T cells contribute to maintaining immune control during LTBI and how HIV perturbs this control are not understood (23). Not all NHPs with LTBI/SIV co-infection exhibit reactivation despite comparable CD4+ T cell depletion (5), indicating that CD4+ T cell-independent mechanisms exist for the maintenance of LTBI control. Co-infection of animals with SIVΔGY showed that CD4+ T cells are important for preventing reactivation during Mtb/SIV. However, the mere depletion of CD4+ T cells is insufficient for reactivation of LTBI, since only 1/8 CD4R1-administered NHPs developed overt TB disease. The key difference in the lungs of primates where lung CD4+ T cells were depleted by antibody or by pathogenic SIV was that the signatures of chronic immune activation and blockade of normal lymphocytic homeostasis were only detected in the lungs of the latter group.

Our results show that lung-specific chronic immune activation may be important for SIV- (and perhaps HIV-) mediated reactivation of LTBI. Since CD4R1 administration
resulted in greater depletion of memory CD4+ T cells and preservation of effector CD4+ T cells, it is possible that this homeostasis drives the lack of reactivation in these NHPs. It is further acknowledged that the lack of chronic immune activation may be a byproduct of the corresponding lack of reactivation. Importantly, it appears that SIV pathogenicity drives mechanisms such as chronic immune activation that disturb the normal homeostasis of effector CD4+ population. Understanding how SIV and chronic immune activation impacts immunity to TB could lead to the development of new treatment paradigms aimed at the cure of Mtb/HIV co-infection.

Methods.

Study approval. All procedures were approved by the TNPRC Institutional Animal Care and Use Committee and Tulane Institutional Biosafety Committee.

Author Contributions. Study design: ANB, SAK, DK; Experiments: ANB, AC, TWF, XA, BT, MGK, NAG; Analysis of results: ANB, AC, TWF, XA, DKS, THL, SAK, DK; Vital reagents/interpretation: JAH, JR, SM, MA; Manuscript preparation: ANB, JR, SM, MA, SAK, DK.
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References.


Figures and figure legends

Figure 1. Comparison of CD4⁺ T cell sparing-SIVmac239ΔGY and antibody-mediated CD4⁺ T cell depletion using CD4R1 in *Mtb*-infected NHPs. (A) Study outline. (B) Serum C-reactive protein (CRP) levels at necropsy. (C) Percent temperature increase (°C) and (D) percent weight loss (kg) compared to baseline. (E) Ratio of neutrophils:lymphocytes in clinical blood. Bacterial burden at necropsy measured in (F) BAL, (G) lung, and (H) bronchial lymph nodes. (I) Chest X-ray scores (CXR) established by veterinary clinicians for pneumonia. (J) Percent lung involvement determined by board-certified veterinary pathologists. (K) Peak plasma viral loads (PVL) measured at peak. *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001, one-way ANOVA with Tukey’s multiple testing correction. C-E represent mean, and B and F-J represent mean ± SEM.
Figure 2. CD4+ T cells are preserved in *Mtb*/SIVΔGY co-infection and ablated in *Mtb*/CD4R1 administration. CD4+ T cells quantification at necropsy SIVΔGY co-infection in (A) peripheral blood, (B) BAL, and (C) lungs. CD4+ T cells quantification in (D) peripheral blood, (E) BAL, and (F) lungs during CD4R1 administration (n=8, red). *P < 0.05; **P < 0.01; ***P <0.001, one-way ANOVA with Tukey’s multiple testing correction. Confocal images of CD4+ cells (red) and nuclei (gray) in formalin-fixed, paraffin-embedded tissue (FFPE) from (G) CD4R1-administered, (H) SIVΔGY co-infected, and (I) SIVmac239 co-infected non-reactivator and (J) reactivator NHPs. (K) Quantification of CD4+ cells per nuclei stained in 9 lung sections from a single slide, one animal per group. Data represent mean ± SEM.
Figure 2.
Figure 3. Peripheral and BAL CD4+ T cells reflect a proportional shift towards effector profile during CD4R1 administration. The proportions of residual effector (CD95+CD28-, circles), memory (CD95+CD28+, squares), and naïve (CD95-CD28+, triangles) (A) CD4+ and (B) CD8+ T cells in the peripheral blood and (C) CD4+ and (D) CD8+ T cells in the BAL of SIVmac239-coinfected (n=15, black) and CD4R1-administered NHPs (n=8, red) compared to LTBI (n=10, blue) at week 11 post-TB. *P < 0.05; **P < 0.01; ***P < 0.001; ****P <0.0001, two-way ANOVA with Tukey’s multiple testing correction. BAL (E) effector and (F) memory CD4+ cells quantification every four weeks starting at week 7 post-TB. Coinfection and antibody administration shown by the dotted line. (G) qRT-PCR analysis of IL-2, IL-12B, and IL-17A collected from BAL cells at week 11 and week 15 post-TB from SIV reactivators (n=3, yellow) and CD4R1-administered NHPs (n=3, red). *P < 0.05; **P < 0.01; ***P < 0.001; ****P <0.0001, multiple T-tests with Holm-Sidak method for multiple comparison correction. Data represent mean ± SEM.
Figure 3.
Figure 4. Immunological analyses of *Mtb/SIV* co-infected and CD4-depleted NHPs reveal reactivation-associated immune impairment. Transcriptomics results generated for necropsy lung tissue (n=3 per group) using (A) InterPro, (B) DAVID Enriched Categories, and KEGG Enriched Pathways. (D) qRT-PCR analysis of TNF-α, IL-1α, IL-1β, and IL-6 from necropsy lung cells of SIV reactivators (n=3, yellow) and CD4R1-administered NHPs (n=3, red). (E) The concentration of TNF-α, IL-1β, and IL-6 measured in the necropsy BAL supernatant of SIV reactivators (n=3, yellow) and CD4R1-administered NHPs (n=5, red). (F) T cell expression of HLA-DR in necropsy lung and co-expression of migratory (CCR5 and CCR7), activation (CD69), and apoptotic (PD-1) markers measured in the (G) HLA-DR+ and (H) HLA-DR- T cell population. *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001, (F) unpaired, two-tailed T-test and (G-H) multiple, unpaired T-tests. Data represent mean ± SEM.
Figure 4.